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Herpes Simplex Virus (HSV)-Specific Proliferative and Cytotoxic T-Cell Responses in Humans Immunized with an HSV Type 2 Glycoprotein Subunit Vaccine

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Studies were undertaken to determine whether immunization of humans with a herpes simplex virus type 2 (HSV-2) glycoprotein-subunit vaccine would result in the priming of both HSV-specific proliferating cells and cytotoxic T cells. Peripheral blood lymphocytes (PBL) from all eight vaccinees studied responded by proliferating after stimulation with HSV-2, HSV-1, and glycoprotein gB-1. The PBL of five of these eight vaccinees proliferated following stimulation with gD-2, whereas stimulation with gD-1 resulted in relatively low or no proliferative responses. T-cell clones were generated from HSV-2-stimulated PBL of three vaccinees who demonstrated strong proliferative responses to HSV-1 and HSV-2. Of 12 clones studied in lymphoproliferative assays, 9 were found to be cross-reactive for HSV-1 and HSV-2. Of the approximately 90 T-cell clones isolated, 14 demonstrated HSV-specific cytotoxic activity. Radioimmunoprecipitation-polyacrylamide gel electrophoresis analyses confirmed that the vaccinees had antibodies only to HSV glycoproteins, not to proteins which are absent in the subunit vaccine, indicating that these vaccinees had not become infected with HSV. Immunization of humans with an HSV-2 glycoprotein-subunit vaccine thus results in the priming of T cells that proliferate in response to stimulation with HSV and its glycoproteins and T cells that have cytotoxic activity against HSV-infected cells. Such HSV-specific memory T cells were detected as late as 2 years following the last boost with the subunit vaccine.

The incidence of genital infections caused by herpes simplex virus type 2 (HSV-2) is increasing (7). Because there is no cure for latent infections induced by this virus (8) and because the disease can be transmitted by asymptomatic individuals (15), there has been much interest in developing a protective vaccine against genital HSV infections. Several HSV vaccine candidates have been developed, including HSV glycoprotein-subunit vaccines (5, 10, 13, 17, 20, 22) and a recombinant vaccinia virus-HSV vaccine (9), which have been found to decrease the likelihood of acquiring disease and latent infections by HSV in experimental animals. One such vaccine candidate is a DNA-free HSV-2 glycoprotein-subunit vaccine, produced from HSV-2-infected-cell extracts, that has been shown to be well tolerated and immunogenic in mice, guinea pigs, and monkeys (10) and has entered human clinical trials (2, 14). We previously reported that humans immunized with this glycoprotein-subunit vaccine developed antibodies capable of neutralizing HSV-2 and mediating antibody-dependent cell-mediated cytotoxicity against HSV-2-infected cells (14). Because T-cell-mediated immune responses, including T-helper cells and cytotoxic T cells (CTL), have been found to play an important role in prevention of and recovery from HSV infections in experimental animals (11, 12, 16, 20), it is likely that vaccine efficacy will require induction of both T-helper cells and CTL in addition to antibodies.

We previously reported that HSV-seropositive humans who are naturally infected with HSV have T cells that proliferate in response to stimulation with glycoproteins gB and gD and that HSV-specific CTL clones can be isolated from such individuals (24–29). We have also found that peripheral blood lymphocytes (PBL) from individuals immunized with the HSV-2 glycoprotein-subunit vaccine can proliferate following stimulation with HSV-2 and with the vaccine preparation (14). There have been varying results as to whether proteins, inactivated viruses, or subunit vaccines can prime CTL in animals (1, 6, 8, 19, 21, 23, 24), and it is not known whether vaccination of humans with a glycoprotein-subunit vaccine can prime virus-specific CTL. This study was undertaken to determine to which HSV glycoprotein(s) the T-cell proliferation responses in these vaccinated humans are directed and also to determine whether this glycoprotein vaccine can prime HSV-specific CTL in humans.

We report here that PBL from all eight vaccinees studied demonstrated proliferative responses to stimulation with HSV-1, HSV-2, and gB-1 and that PBL from five also responded to gD-2. HSV-specific CTL clones were isolated from vaccinees who showed no serological evidence of having been infected with HSV, thus indicating that a glycoprotein-subunit vaccine can also prime virus-specific CTL in humans.

MATERIALS AND METHODS

Vaccinations of humans. The HSV-2 glycoprotein-subunit vaccine (lot 806; Merck Sharp & Dohme Research Laboratories, West Point, Pa.) was prepared as previously described (10). Briefly, HSV-2-infected chicken embryo fibroblasts were disrupted with detergent, treated with DNase, and subjected to chromatography to select for glycoproteins. The vaccine was then treated with Formalin, and alum was added as adjuvant. Vaccine (50 μg per dose) was administered as previously described (9) at days 0, 28, and 140 to healthy adults who were shown to be HSV seronegative by complement-independent neutralization and by radioimmu-

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noprecipitation-polyacrylamide gel electrophoresis (RIP-PAGE) (2). PBL from the vaccinees were isolated between 6 months and 2 years following their third vaccination. PBL were isolated from vaccinees from whom T-cell clones were generated at 22, 24, and 7 months, respectively, for individuals A, B, and C. PBL were also isolated from controls, including an individual, D, who had recurrent HSV-2 genital infections approximately every 8 weeks and was HSV-2 seropositive and from individual E, who suffered recurrent HSV-1 oral lesions and was HSV-1 seropositive. PBL were also isolated from F, an HSV-seronegative individual who had no history of oral lesions or genital HSV infections. Sera from blood from which PBL had been isolated were retained to test for antibodies to wild-type HSV proteins.

Radioimmunoassay for the detection of HSV antibodies. Sera from the vaccine recipients and control sera from an HSV-seronegative donor and an HSV-2-seropositive donor were analyzed by RIP-PAGE as previously detailed (2, 4). Briefly, HSV-1 (strain E115)- or HSV-2 (strain 33)-infected-cell proteins were radiolabeled with [^{35}S]methionine (New England Nuclear Corp., Boston, Mass.) and harvested as cytoplasmic extracts as described previously (4). Sera were diluted 1:4 and incubated with the radiolabeled proteins, which were diluted 1:40 in 0.01 M Tris (pH 8.0). Antigen-antibody complexes were collected on SPA-Sepharose beads (Sigma Chemical Co., St. Louis, Mo.), washed, and electrophoretically separated on 9% polyacrylamide gels with 1% sodium dodecyl sulfate. Gels were soaked in 1 M sodium salicylate and exposed to Kodak X-ray film for 5 days.

Viruses. The KOS strain of HSV-1 and the 186 strain of HSV-2, both grown in HEp-2 cells, were kindly provided by S. Tevethia, Pennsylvania State University, Hershey, Pa. UV light-irradiated purified HSV-1 and HSV-2 were used for the stimulation of PBL and the maintenance of T-cell clones, and live HSV-1, HSV-2, and vaccinia viruses were used to infect lymphoblastoid cell lines (LCL) for use as target cells.

Cell lines. LCL were established in our laboratory by transforming PBL from the vaccinees with Epstein-Barr virus, as described previously (25). The LCL were maintained in RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum.

Stimulation of PBL with HSV and HSV glycoproteins. PBL were separated from heparinized blood by Ficoll-Hypaque gradient centrifugation, and 5×10^6 PBL were suspended in 20 ml of RPMI 1640 medium supplemented with 15% heat-inactivated normal human serum (this medium is referred to as complete medium). Then, 5×10^4 cells were added to each of four replicate round-bottomed wells, followed by the addition of UV light-irradiated HSV-1 or HSV-2 (10^6 PFU/ml prior to UV irradiation). In addition, PBL were stimulated with 0.7 μg of purified gB-1, gD-1, and gD-2 per ml, which were cloned and expressed in the mammalian CHO cells as previously reported (26, 27). Seven days after the onset of stimulation, 1 μCi of [^{3}H]thymidine ([^{3}H]TdR; New England Nuclear) was added to each of four replicate wells for the last 6 h of incubation at 37°C. Cells were harvested by using a multiple well-harvesting apparatus, and [^{3}H]TdR incorporation was determined by liquid scintillation counting.

Generation of T-cell clones. PBL were stimulated twice at weekly intervals with UV light-inactivated HSV-2 in complete medium. Three days after the second stimulation, single cells were cloned by limiting dilution in microwells containing 5×10^4 X-irradiated (2,500 R) autologous PBL as antigen-presenting cells, UV-inactivated HSV-2, and human interleukin-2 (IL-2; Cellular Products, Buffalo, N.Y.) in complete medium as we previously detailed (25, 26). The clones were expanded to 24-well plates and were fed every 10 days with 2.5×10^5 X-irradiated autologous PBL, UV-inactivated HSV-2, and IL-2-containing complete medium. Most clones were tested two or three times for their ability to proliferate following stimulation with HSV-1, HSV-2, or no antigens in IL-2-free medium and for their ability to lyse ^{51}Cr-labeled virus-infected cells, as described below.

^{51}Cr-release assays. ^{51}Cr-release assays were performed as previously described (15). To prepare virus-infected target cells, 3×10^6 LCL were infected with 3×10^7 PFU of HSV-1, HSV-2, or vaccinia virus in serum-free RPMI 1640 medium for 1 h. The cells were then cultured overnight in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, labeled with ^{51}Cr (Na_2CrO_4; New England Nuclear), and washed three times with medium. A total of 3×10^2 ^{51}Cr-labeled target cells were then placed into four replicate round-bottomed microdilution wells containing HSV-stimulated noncloned effector cells or cloned effector cells in a final volume of 0.2 ml of medium per well, resulting in an effector/target cell ratio of 25:1. In some experiments, lysis was also measured at effector/target cell ratios of up to 100:1. Target cells were also added to six wells containing medium alone and to six wells containing detergent to determine spontaneous and maximal ^{51}Cr release, respectively. After 6-h incubations, 0.12 ml of supernatant was removed from each well and transferred to tubes for counting in a gamma counter. The percent specific ^{51}Cr release was calculated as follows: [(cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release)] × 100. The spontaneous release from the target cells did not exceed 20% of the maximal release.

RESULTS AND DISCUSSION

Eight recipients of an HSV-2 glycoprotein-subunit vaccine whose sera had been shown by RIP-PAGE to contain antibodies to HSV glycoproteins but not to nonglycosylated proteins, indicative of naturally acquired HSV infection (2, 3), were selected for these studies on HSV-specific T-cell immunity. Results (Table 1) show that PBL from all eight vaccinees studied demonstrated strong proliferative responses to UV-inactivated HSV-2 and HSV-1, as did PBL from the two naturally infected HSV-seropositive donors, even though the vaccinees had received their last vaccine boost 6 to 24 months previously. The HSV-seronegative donor demonstrated no proliferative responses to HSV. PBL from all eight vaccinees also demonstrated proliferative responses to gB-1, consistent with the fact that gB is a highly type-common protein, and PBL from five of the eight also demonstrated proliferative responses to gD-2. In this study (Table 1) and in our previous studies (29), PBL from individuals naturally infected with HSV showed strong proliferative responses to gB-1 and gD-1 and significant responses to gD-2. In contrast, the response of PBL from the recipients of the HSV-2 glycoprotein-subunit vaccine to gD-1 was absent in four of the eight vaccinees and relatively weak in the others.

Vaccinees A, B, and C, who showed strong proliferative responses to both HSV-1 and HSV-2, were chosen for further study to evaluate HSV type specificity of proliferative and cytotoxic responses on a clonal level. Results (Table 2) show that, of eight clones from vaccinee A studied, six responded by proliferating to both HSV-1 and HSV-2, while

TABLE 1. Proliferative responses of PBL from HSV-subunit-vaccinated and HSV naturally infected individuals[a]

Individual	Proliferative response (stimulation index)[b] to:				
	UV-HSV-1	UV-HSV-2	gB-1	gD-1	gD-2
Vaccinees					
A	42.9	109.0	67.2	2.3	145.0
B	54.7	97.7	88.8	1.1	16.0
2573	18.3	80.6	11.8	6.8	1.7
2070	54.7	100.0	156.7	1.0	0.7
C	31.6	34.6	19.8	1.1	2.8
2348	25.4	58.6	49.5	3.9	20.2
3074	21.4	37.7	22.0	6.8	0.5
2926	3.5	33.6	6.2	0.6	7.1
HSV Seropositive					
D	90.8	144.5	73.1	32.2	3.9
E	41.5	75.2	27.9	20.7	7.9
HSV Seronegative (F)	0.1	2.0	1.4	0.4	0.2

[a] PBL from individuals immunized with the HSV-2 glycoprotein vaccine, from HSV-seropositive, naturally HSV-infected individuals (D and E), and from an HSV-seronegative individual (F) were stimulated for 6 days with UV-inactivated HSV-1 or HSV-2 (UV-HSV-1 and UV-HSV-2, respectively) or with purified cloned glycoprotein gB-1, gD-1, or gD-2 (0.7 μg/ml) prior to labeling for 6 h with [^{3}H]TdR.

[b] Stimulation index = counts per minute of [^{3} H]TdR incorporated into antigen-stimulated cells/counts per minute of [^{3}H]TdR incorporated into nonstimulated cells.

two were HSV-2 type-specific. Of the four clones tested from vaccinee C, three were HSV-1 and HSV-2 cross-reactive.

While we and others have previously reported that humans and mice infected with HSV have HSV-specific CTL (11, 12, 16, 20, 25–29), it has not previously been reported whether immunization of humans with a viral glycoprotein-subunit vaccine can induce virus-specific CTL. Thus, experiments were performed to determine whether HSV-specific CTL are also primed in individuals immunized with the

TABLE 2. HSV-induced proliferative responses of T-cell clones isolated from recipients of an HSV-subunit vaccine[a]

Vaccinee/clone no.	cpm of [^{3}H]TdR incorporation (± SD)[b] induced by:		
	No virus	HSV-2	HSV-1
A			
3	37 ± 19	920 ± 143	535 ± 222
5	105 ± 99	7,927 ± 1,919	12,537 ± 676
8	180 ± 80	7,332 ± 1,201	5,837 ± 1,055
12	170 ± 29	13,555 ± 3,112	397 ± 226
19	57 ± 17	1,605 ± 451	85 ± 21
29	75 ± 29	6,805 ± 2,041	11,945 ± 2,656
36	40 ± 34	4,375 ± 1,597	70 ± 41
39	40 ± 18	2,781 ± 1,321	130 ± 56
C			
2	40 ± 18	580 ± 122	145 ± 21
3	50 ± 38	23,530 ± 3,827	83 ± 44
4	38 ± 38	10,985 ± 3,355	6,090 ± 1,567
8	597 ± 119	2,117 ± 555	2,067 ± 555

[a] PBL were stimulated with UV-inactivated HSV-2 and clones were generated as described in Materials and Methods. The clones were stimulated with UV-inactivated HSV-1, HSV-2, or no antigen in the absence of exogenous IL-2, and [^{3}H]TdR incorporated into cloned cells was measured 3 days later.

[b] Data shown are mean counts per minute of [^{3}H]TdR incorporated into cells in four replicates ± the standard deviation of the mean.

TABLE 3. HSV-specific CTL clones from recipients of an HSV glycoprotein-subunit vaccine[a]

Vaccinee/clone no.	% Specific lysis of autologous cells infected with:		
	HSV-2	HSV-1	Vaccinia virus
C			
4	46.4	17.5	1.0
6	22.2	6.4	−5.2
8	31.0	15.5	0.4
10	25.2	6.1	−5.2
13	19.0	−0.2	−0.5
14	29.5	10.4	0.5
Bulk (noncloned)	70.1	49.6	39.6
A			
16	13.4	3.3	−3.2
19	11.3	2.1	−1.6
25	9.9	−0.9	−0.3
36	16.9	2.6	−1.2
Bulk (noncloned)	42.6	33.2	24.7
B			
9	10.3	−0.2	−0.5
12	10.6	8.1	−1.7
21	26.4	22.9	−0.6
24	15.2	17.9	−1.4

[a] PBL from the vaccinees were stimulated with UV-inactivated HSV-2, and clones were generated as described in Materials and Methods. Clones were tested for cytotoxic activity against ^{51}Cr-labeled autologous lymphoblastoid cells infected with HSV-1, HSV-2, or vaccinia virus as detailed in Materials and Methods by using an effector-to-target cell ratio of 25:1.

TABLE 4. HSV-specific CTL clones from recipients of an HSV-2 glycoprotein-subunit vaccine at different effector-to-target cell ratios

Vaccinee/clone no.	% Specific lysis of autologous cells infected with HSV-2 at an E:T ratio[a] of:		
	100:1	50:1	25:1
C (8)	55.4	41.3	21.4
A			
16	52.5	39.4	28.5
36	36.0	33.5	17.8
Bulk	32.7	29.1	25.2

[a] E:T ratio, Effector-to-target cell ratio.

HSV-2 glycoprotein-subunit vaccine. Because HSV-stimulated noncloned cultures contain natural killer-like cells which can lyse cells infected with a variety of viruses and which can mask HSV-specific CTL responses (25), we generated T-cell clones from the three vaccinees. A total of approximately 90 clones generated by stimulating PBL with HSV-2 were tested for their ability to lyse autologous LCL infected with HSV-1, HSV-2, or unrelated vaccinia virus. Table 3 lists the clones that mediated HSV-specific cytotoxicity at effector-to-target cell ratios of 25:1. Whereas HSV-2-stimulated noncloned cells growing in IL-2 were cytotoxic for HSV-1-, HSV-2-, and vaccinia virus-infected autologous cells, owing to the presence of natural killer-like cells, 14 clones were isolated from these individuals which were found to be either HSV-2 specific or HSV-1 and HSV-2 cross-reactive and which lacked cytotoxic activity against cells infected with vaccinia virus. It is likely that higher levels of cytotoxicity were not observed because of the narrow window of time between expression of HSV glyco-

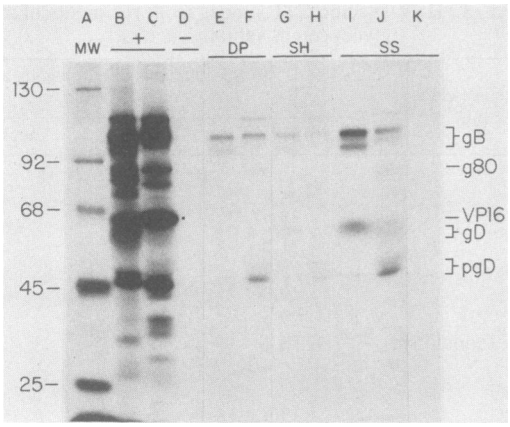

FIG. 1. RIP-PAGE analysis of sera for HSV-specific antibodies. Sera from vaccine recipients A (DP), B (SH), and C (SS) were reacted with radiolabeled HSV-1-infected cell proteins (lanes E, G, and I) or with HSV-2-infected-cell proteins (lanes F, H, and J) as described in Materials and Methods. In addition, serum from C was reacted with mock-infected cells (lane K). Control serum from a seropositive patient (+) was reacted with the HSV-1 preparation (lane B) and the HSV-2 preparation (lane C). Serum from a seronegative individual (−) was reacted with the HSV-2 preparation (lane D). Molecular sizes of size standards (lane A) are indicated in kilodaltons. HSV-2 proteins were identified by migration characteristics as indicated on the right. Antibody profiles of vaccinees are limited to viral glycoproteins, while those of the seropositive control contain numerous bands, including a sharp dense band (VP16) which has been shown to be a useful marker of natural infection (3) as it is not contained in the vaccine.

proteins on the surface of HSV-infected LCL and the subsequent death of the LCL. Therefore, cytotoxicity experiments had to be carried out before all of the cells expressed HSV antigens on their surfaces. However, we did observe higher levels of cytotoxicity when the ratio of cloned effector cells to target cells was increased above 25:1 (Table 4).

The cytotoxic and proliferating clones which were phenotyped with regard to expression of T-cell markers were found to be $CD4^+$, with the exception of one that was $CD4^-$ $CD8^+$ and one that was $CD4^+CD8^+$. This finding, that most clones were $CD4^+$, is in agreement with our previous observations that HSV-specific CTL clones isolated from HSV-1- and HSV-2-seropositive, naturally infected individuals are $CD4^+$ (25, 29) and restricted by HLA class II antigens (25). While we did not carry out studies to define the nature of HLA restriction of the CTL clones isolated from the vaccinees, we did observe that the clones described did not lyse HSV-2-infected allogeneic target cells sharing no HLA class I or class II antigens with PBL of the clone donors. Since LCL express both HLA class I and II antigens, their use as targets should not preclude the detection of $CD8^+$, HLA class I-restricted CTL. We also attempted to use autologous phytohemagglutinin-stimulated PBL infected with HSV-2 as targets; however, we observed that a lower percentage of PHA blasts expressed HSV cell surface antigens than did LCL and are less suitable as target cells.

Because individuals can become infected with HSV-2 and remain asymptomatic (15), it was necessary to rule out that the vaccinees from whom we generated CTL clones had become infected with HSV-2. Results of RIP-PAGE analysis of sera from these individuals are shown in Fig. 1. Serum antibodies of the vaccinees were limited to those proteins present in the subunit vaccine, including gB (seen in all three vaccinees), g80 (a complex of glycoprotein C and E seen in vaccinee C only), gD (seen in vaccinee C only), and the precursor to gD, pgD (seen in all three vaccinees). Serum antibodies to nonglycosylated proteins (i.e., VP16, which is present in HSV but absent in the subunit vaccine) were not detected in the vaccinees' sera; however, they are readily detectable in individuals who are infected with HSV (Fig. 1) (3).

Our results thus enable us to conclude that immunization of humans with an HSV-2 glycoprotein-subunit vaccine results not only in serological responses, as previously reported (2, 14), but also in the priming of T cells which proliferate in response to stimulation with HSV-1, HSV-2, and HSV glycoproteins and in the priming of HSV-specific CTL. Both proliferative and cytotoxic T-cell responses to HSV-2 could be detected as late as 2 years after the last boost with the subunit vaccine. It is important to mention, however, that only 14% of the approximately 90 clones isolated from the vaccinees were cytotoxic for autologous HSV-infected cells. This contrasts with our previous finding, that approximately 50% of the HSV-specific T-cell clones which we isolated from HSV-seropositive, naturally infected donors have HSV-specific CTL activity (25–29). Thus, the proportion of HSV-specific T cells that have cytotoxic activity appears to be lower in the recipients of the glycoprotein-subunit vaccine than we previously observed in naturally infected individuals. This observation is similar to that of previous reports, that while inactivated influenza and herpesviruses and influenza subunit vaccines are effective in inducing antivirus antibodies, they are relatively poor, as compared with live virus, at priming virus-specific CTL in mice (1, 6, 18, 19, 24). It is possible that the apparent relatively low level of priming of HSV-specific cytotoxic T cells in humans following vaccination with this HSV-2 glycoprotein-subunit vaccine may be related to our finding, to be reported elsewhere, that this vaccine does not appear to be protective against subsequent acquisition of HSV-2 infections.

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